Control of cell growth by c-Myc in the absence of cell division

Marino Schuhmacher*, Martin S. Staege*, Alexander Pajic*, Axel Polack*, Ulrich H. Weidle†, Georg W. Bornkamm*, Dirk Eick* and Franz Kohlhuber*

The c-Myc protein (Myc) is a transcription factor, and deregulated expression of the c-*myc* gene (*myc*) is frequently found in tumours. In Burkitt's lymphoma (BL), *myc* is transcriptionally activated by chromosomal translocation. We have used a B-cell line called P493-6 that carries a conditional *myc* allele to elucidate the role of Myc in the proliferation of BL cells. Regulation of proliferation involves the coordination of cell growth (accumulation of cell mass) and cell division [1–3]. Here, we show that division of P493-6 cells was strictly dependent on the expression of the conditional *myc* allele and the presence of foetal calf serum (FCS). More importantly, cell growth was regulated by Myc without FCS: Myc alone induced an increase in cell size and positively regulated protein synthesis. An increase in protein synthesis is thought to be one of the causes of cell mass increase. Furthermore, Myc stimulated metabolic activities, as indicated by the acidification of culture medium and the activation of mitochondrial enzymes. Our results confirm the model that Myc is involved in the regulation of cell growth [4] and provide, for the first time, direct evidence that Myc induces cell growth, that is, an increase in cell size, uncoupled from cell division.

Addresses: *GSF Research Centre, Institute of Clinical Molecular Biology, Marchioninistraße 25, D-81377 Munich, Germany. †Roche Diagnostics GmbH, Roche Pharma Research Penzberg, Department TR-0N, 82372 Penzberg, Germany.

Correspondence: Franz Kohlhuber
E-mail: kohlhuber@gsf.de

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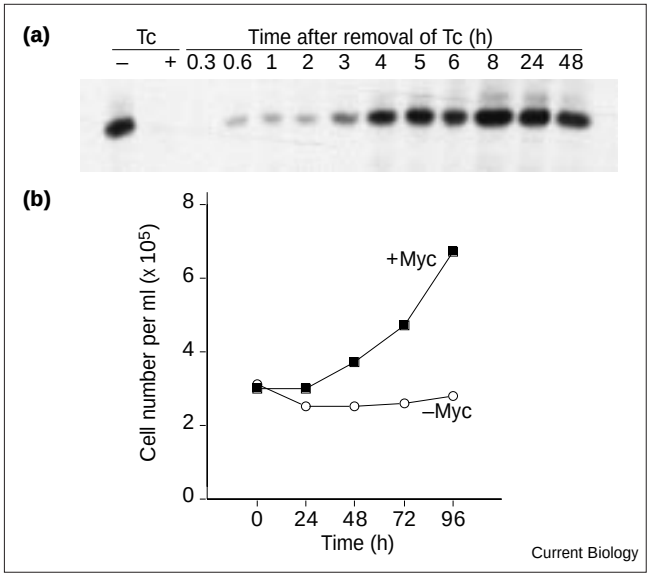
Results and discussion

We have shown previously that constitutive expression of *myc* in human B lymphocytes transformed with a conditionally active Epstein–Barr virus (EBV) [5] allows proliferation in the absence of EBV functions [6]. Here, we have extended these experiments and expressed a conditional tetracycline (Tc)-regulatable [7] *myc* construct in the P493-6 B-cell line. In these cells, *myc* was expressed in the absence of Tc and reached levels comparable to other BL cell lines, but was not expressed in the presence of Tc (Figure 1a; A. Pajic, D. Spitkovsky, B. Christoph, B. Kempkes, M. Schuhmacher, M.S. Staege, *et al.*, unpublished observations). Proliferation of P493-6 cells was strictly dependent on the expression of *myc* (Figure 1b). Tc had no effect on the proliferation of the parental cell line (data not shown).

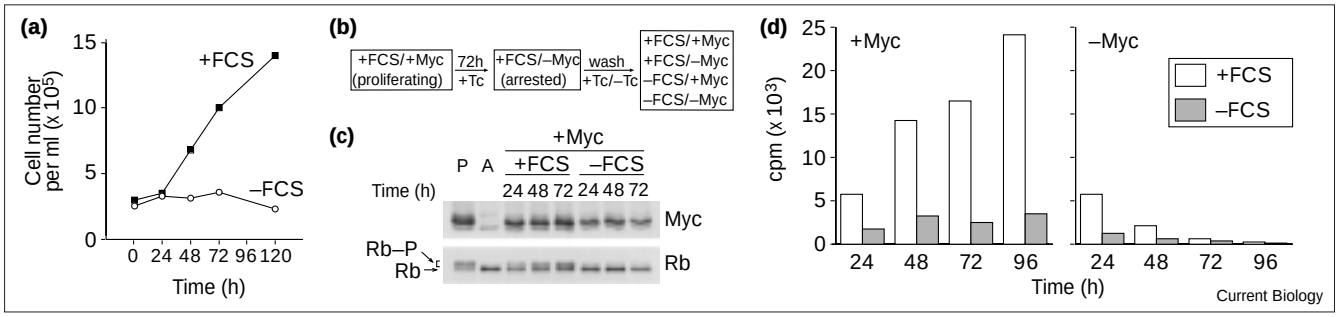
Activation of a conditionally active Myc protein in quiescent fibroblasts triggers progression through the cell cycle [8] and hyperphosphorylation of the retinoblastoma protein (Rb) [9]. In P493-6 cells, however, *myc* expression did not induce either proliferation (Figure 2a) or hyperphosphorylation of Rb (Figure 2b,c) in the absence of

Figure 1



Characterization of P493-6 cells. All experiments were performed in the presence of 10% FCS. (a) Regulation of the conditional *myc* allele by Tc. Levels of Myc protein were monitored by western-blot analysis using the anti-Myc antibody 9E10 (Santa Cruz Biotechnology). Myc protein was expressed in untreated cells (–); after treatment with 0.1 μg/ml Tc for 72 h (+), Myc protein could no longer be detected. Subsequent removal of Tc by washing the cells with PBS allowed detection of Myc within 40 min in a time-course experiment. (b) Proliferation of P493-6 cells is dependent on the expression of *myc*. P493-6 cells were arrested in the G1 phase of the cell cycle by adding Tc for 72 h (data not shown). Subsequently, half the cells were washed with PBS to remove Tc (to turn on expression of *myc*) and seeded into culture medium without Tc; the other half were washed with PBS in the presence of Tc and cells were seeded into culture medium containing Tc. Cell numbers were determined every day; 0 h, cell number determined immediately after washing the cells; +Myc, *myc* is expressed; –Myc, *myc* is not expressed.

Figure 2



Proliferation of P493-6 cells is dependent on the expression of *myc* and the presence of FCS. (a) Proliferating cells were washed four times with PBS to remove FCS; half the cells were seeded into culture medium containing 10% FCS (+FCS) and the other half were transferred into medium containing 0.25% FCS (–FCS). Cell numbers were determined every day; 0 h, cell number determined immediately after washing the cells. Re-addition of FCS to starved cells re-induced proliferation (data not shown). (b) Schematic overview of the experimental procedure. P493-6 cells were arrested in G1 phase by addition of Tc for 72 h in the presence of FCS. Subsequently, half the cells were washed in PBS to remove FCS and Tc and thus to induce *myc* expression (+Myc); the other half were washed in the presence of Tc (no *myc* expression; –Myc). Half the cells that were washed without Tc were transferred to culture medium containing 10% FCS (+FCS) and the other half were transferred to medium containing only 0.25% FCS (–FCS). Cells washed in the presence of Tc were processed identically except that Tc was added to the culture media. (c) Phosphorylation of Rb. Cells were processed as outlined in (b) Whole-cell extracts (normalised for cell numbers) were prepared 24 h, 48 h and 72 h after washing and analysed for Myc and Rb levels by western blotting. Antibodies were from Santa Cruz Biotechnology. P, proliferating P493-6 cells; A, arrested cells; Rb, hypophosphorylated form of Rb; Rb–P, hyperphosphorylated and inactive form of Rb. (d) Incorporation of [^{3}H]thymidine. Cells were processed as outlined in (b). Cells were counted every day and the same number of cells was incubated in the appropriate medium with [^{3}H]thymidine for 24 h intervals: 0–24 h (24 h), 24–48 h (48 h), 48–72 h (72 h) and 72–96 h (96 h). Cells were harvested using the Canberra Packard Filtermate 196 Harvester. Incorporation of radioactivity in counts per minute (cpm) was determined using the Canberra Packard Topcount Microplate Scintillation Counter.

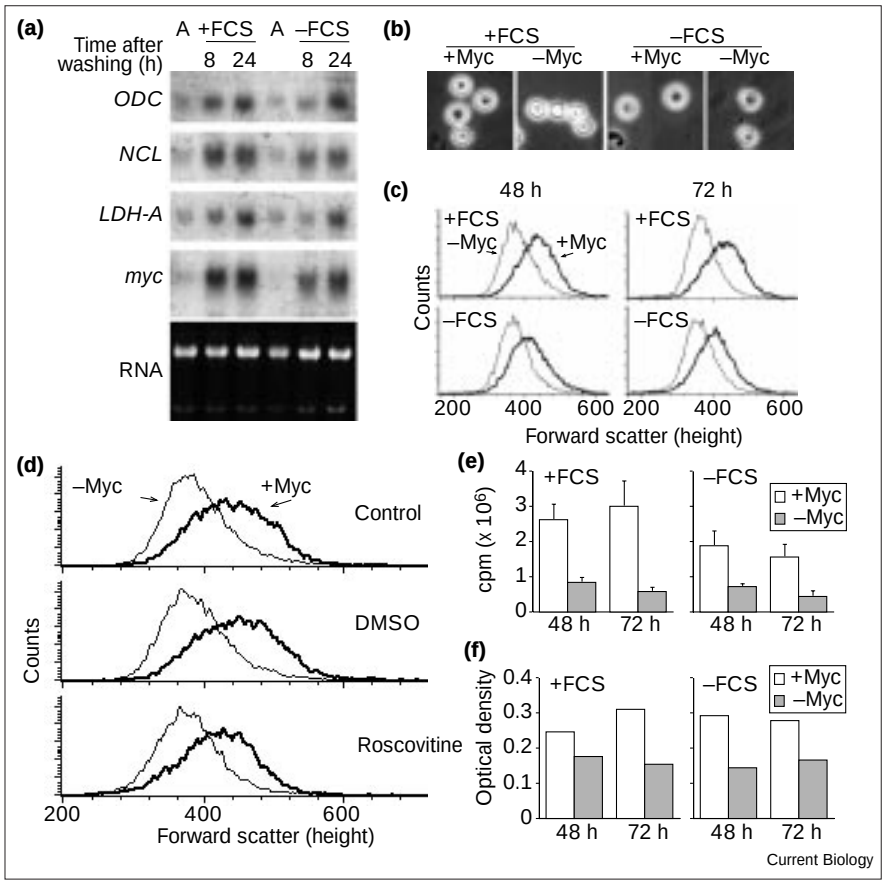
FCS. Incorporation of [^{3}H]thymidine, which labels cells progressing through S phase, was substantially decreased in the absence of FCS (Figure 2d). Similar results were obtained by flow-cytometric cell-cycle analysis (data not shown). As the expression of *myc* appeared to be decreased in the absence of FCS (Figure 2c), we cannot rule out the possibility that Myc levels were not sufficient to induce phosphorylation of Rb. More importantly, we did not observe Myc-induced apoptosis [10] in the absence of FCS (data not shown).

Although Myc did not induce proliferation in the absence of FCS, we found Myc-dependent activities and phenotypes. Transcription of known Myc target genes such as ornithine decarboxylase [11], lactate dehydrogenase A [12] and nucleolin (M. Schuhmacher and D. Eick, unpublished observations; [13]) was induced by Myc in P493-6 cells in the presence and absence of FCS (Figure 3a). We discovered the most striking FCS-independent function of Myc simply by microscopic inspection of our cells: whenever *myc* was expressed, the average size of the P493-6 cells was increased compared with cells that did not express *myc* (Figure 3b). This observation was confirmed by flow-cytometric forward-scatter analysis (Figure 3c). To further support our hypothesis that the effect of Myc on cell growth is independent of its effect on cell division, we tested whether P493-6 cells still grew after *myc* induction in the presence of roscovitine, a specific inhibitor of cyclin-dependent kinase 2 [14]. Treatment of P493-6 cells with roscovitine in the presence of FCS blocked phosphorylation of Rb and cell-cycle entry (data not shown). The increase in cell size, however, was similar to that of the untreated control cells (Figure 3d).

Cell growth most probably occurs as a result of an increase in cell mass due to protein synthesis. In accordance with our data regarding cell size, incorporation of labelled amino acids into trichloroacetic acid (TCA)-precipitable protein was clearly elevated in *myc*-expressing P493-6 cells (Figure 3e). Tc had no effect on cell size or protein synthesis in the parental cells (data not shown). A Myc-dependent increase in protein synthesis has also been observed in fibroblasts carrying a conditional *myc* allele [4,15]; it was not shown that this results in an increase in cell size, however. Expression of known Myc target genes that appear to be required for protein synthesis was induced by Myc in P493-6 cells, for example, translation initiation factor eIF-4E [16] (data not shown) and the nucleolar protein nucleolin (Figure 3a).

Two further observations suggest that Myc regulates energy metabolism, another growth-related activity. The culture medium of P493-6 cells that expressed *myc* in the absence of FCS turned slightly yellow with time due to acidification (data not shown). As shown by northern-blot analysis (Figure 3a), transcription of the LDH-A gene

Figure 3



Myc-dependent and FCS-independent phenotypes. P493-6 cells were processed as outlined in Figure 2b. (a) Northern-blot analysis of putative Myc target genes. Total RNA was extracted using an RNeasy kit (Qiagen) from arrested cells (A) as well as 8 h and 24 h after washing the cells. The expression of the following genes was monitored using ^{32}P-labelled cDNA probes: ornithine decarboxylase (*ODC*), nucleolin (*NCL*), lactate dehydrogenase (*LDH-A*) and *myc*. The ethidium-bromide-stained gel at the bottom shows the loading of RNA. (b–d) Myc increases the average cell size. (b) Photographs taken 72 h after washing the cells. (c) Flow-cytometric forward-scatter (FSC) analysis of viable, propidium-iodide-negative P493-6 cells, 48 h and 72 h after washing. The FSC analyses were performed individually; subsequently, the individual histograms were overlaid as shown. (d) FSC analysis of roscovitine-treated cells in the presence of FCS. After the arrested cells had been washed with PBS with or without Tc, they were transferred for 72 h into medium with FCS without or with Tc (+Myc and –Myc, respectively; control); 30 μM roscovitine (dissolved in dimethyl sulfoxide, DMSO) or DMSO was added as indicated. FSC analyses were performed as in (c). (e) Protein synthesis 48 h and 72 h after washing the cells (three independent experiments). Cells (3×10^6) were labelled in 3.5 ml methionine- and cysteine-free medium in the presence of 0.25% (–FCS) or 10% (+FCS) dialysed FCS with or without Tc and with 200 μCi [^{35}S] Tran-S-Label (ICN) for 1 h at 37°C. Subsequently, cells were washed and lysed. Equal aliquots of the extracts were precipitated with TCA on glass microfibre filters (GF/C; Whatman) and TCA-insoluble counts per min were determined as described [19]. (f) MTT assay [5], which measures activation of mitochondrial enzymes. At 48 h and 72 h after the PBS wash, 3×10^4 cells were seeded in 100 μl of the appropriate medium in 96-well plates and incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2 2,5-diphenyl tetrazolium bromide; 0.5 mg/ml; Sigma) for 4 h at 37°C. After incubation, 200 μl 1 N HCl/isopropanol (1:24) was added to all wells and mixed thoroughly to dissolve the formazan crystals. The optical density of the samples was read on an enzyme-linked immunosorbent assay reader. The results of a representative experiment are shown.

was induced by Myc. This could produce lactic acid, explaining the acidification. We also observed Myc-dependent activation of mitochondrial enzymes in the MTT assay [5] (Figure 3f).

The involvement of Myc in metabolism and macromolecular synthesis has been suspected from studies of fibroblasts [4,15] and putative Myc target genes [17,18] as well as from analyses of a *myc* knock-out cell line [19]. The results of all these studies have recently been summarized and reviewed by Schmidt [4]. Our data support and strengthen the concept that Myc is a regulator of cellular growth. Moreover, compared with the fibroblast systems, our B cells showed a significant Myc-dependent increase in cell size, which would be expected as a consequence of an increase in protein synthesis. In addition, as postulated by Schmidt [4], the enlargement of P493-6 cells could be uncoupled from cell division, indicating that Myc increases cell growth independently of its effects on cell division. Interestingly, B cells from mice expressing *myc* under the control of the Eμ immunoglobulin enhancer were significantly larger than B cells from littermate controls (B. Iritani and R.N. Eisenman, unpublished observations).

The growth-promoting function of Myc does not appear to be restricted to mammalian cells. A homolog of mammalian *myc*, *dmyc*, has been cloned in *Drosophila* [20,21]. Recent results of analyses in *Drosophila* imaginal discs [22] have shown that *dmyc* loss-of-function mutations result in smaller cells and overexpression of *dmyc* results in larger cells without a change in cell-doubling time. Therefore, *dmyc* also appears to influence the growth of cells in *Drosophila*.

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